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[^{3}H]UTP INCORPORATION IN PERMEABILIZED MOUSE L-CELLS IS INHIBITED BY ANTIBODIES DIRECTED AGAINST A STRUCTURAL NUCLEAR PROTEIN

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SUMMARY: Newly transcribed RNA is apparently attached to a nuclear structural framework whose main proteins are lamins A, C and B. Whether this attachment is fortuitous or has some functional importance was tested by measuring [^{3}H]UTP incorporation into RNA of permeabilized mouse L-cells after incubation with antibodies against these polypeptides. Antibodies against only lamin B and not against lamins A,C inhibited low concentration α-amanitin-sensitive RNA transcription. Lamin B antibodies inhibited only in an assay system with whole nuclei and not in a soluble assay system. Anti-lamin B therefore does not block the actual transcriptional machinery, but rather seems to crosslink the structural framework on which transcription takes place in the intact nucleus, inhibiting dynamic changes essential to RNA synthesis.

Structural nuclear proteins are presently being identified, characterized and investigated for their functional properties. In particular, proteins which are present in the nuclear envelope residue called fibrous lamina, or in the nuclear matrix, operationally defined as the residue of a rather harsh extraction regimen (1-5), are being defined as to their location and physical properties (6, 7). The major proteins or lamins (1) have been considered only as structural proteins at the periphery of the nucleus, with a possible function in the attachment of chromatin to the nuclear envelope (8). However, recent evidence that specific products of RNA synthesis are enriched on the matrix, that enzymes of DNA synthesis are preferentially bound to the matrix (9, 10), and that antibodies to nuclear envelope lamin B prevent RNP efflux in an in vitro system (11) suggest a variety of functions which could be attributed to those structures of the nuclear framework. The involvement

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Abbreviations: PBS, phosphate-buffered saline; UTP, uridine triphosphate; TCA, trichloroacetic acid; Ig, immunoglobulin; NTPase, nucleotide triphosphatase; NTP, nucleotide triphosphate; RNP, ribonucleoprotein; DEAE, diethyl aminoethyl.

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of certain proteins in activities for which assays exist could be studied if antibodies were available that interact specifically with these protein antigens. Antibody reduction during RNP release, NTPase activity (11) or insulin-stimulated RNP release (12) indicate that such experiments are feasible. In the present study, we document our finding that antibodies to lamin B reduce the level of [^{3}H]uridine incorporation in permeabilized cultured cells.

MATERIALS AND METHODS

Cell line: Mouse L-cells were plated weekly at a 1:4 split ratio. Cells were grown at 37°C in a 5% CO_2 atmosphere in Auto-Pow MEM supplemented with 10% fetal bovine serum.

Preparation of permeabilized cells: Cells growing in T-75 Falcon flasks containing 10 ml medium were prelabeled for 2 h with 0.2 µCi/ml of [^{14}C]thymidine (spec. act. 53.4 mCi/mmol) and followed by a 2-h chase with fresh prewarmed medium. Permeabilized mouse L-cells were prepared by a modification of the procedure of Rovera and co-workers (13). The cell monolayers were washed twice with cold PBS without Ca^{++} and Mg^{++}, and treated with 2.5 ml of medium K (10 mM Tris, pH 7.6, 6 mM magnesium acetate, 125 mM KCl, 6 mM β-mercaptoethanol) containing 0.5% Triton X-100. The medium was left on the cell for 2 min and removed. Medium K was added to the permeabilized cell monolayer and the cells were shaken off. They were collected by gentle centrifugation (5 min at 700 g) and resuspended in buffer containing 50 mM Tris-HCl, pH 8.5, 2.5 mM $MgCl_2$, and 2 mM dithiothreitol.

Assay for UTP incorporation: The assay mixture for endogenous polymerase activity (final volume 0.2 ml) consisted of 0.05 ml of permeabilized cell suspension (~ 5 x 10^{-5} cells), 50 mM Tris-HCl, pH 8.5, 2.5 mM $MgCl_2$, 2 mM dithiothreitol, 0.2 mM ATP, 0.2 mM CTP, 0.2 mM GTP and 10 µCi [^{3}H]UTP (51 Ci/mmol). After a 10-min incubation with antibodies, the permeabilized cells were washed once and incubated at 32°C in a water bath and the reaction terminated by the addition of 100 µl of 10% TCA containing 50 mM sodium pyrophosphate. The acid-insoluble radioactivity was collected on Whatman GF/C filters. These filters were washed three times with 10 ml of cold 5% TCA containing 5 mM sodium pyrophosphate. The samples were counted and the cpm corrected for spillover of ^{14}C into the ^{3}H channel. The counts were expressed as cpm [^{3}H]UTP incorporated into RNA per cpm [^{14}C]thymidine incorporated into DNA.

For in vitro transcription without nuclei we used a HeLa cell lysate transcription system with adenovirus SmaI-F fragment as the DNA template (14). Reaction mixtures (25 µl) contained 60 mM KCl, 10 mM HEPES, pH 7.9, 7.5 mM $MgCl_2$, 2 mM DTT, 4 mM $_2$ creatine phosphate, 500 µM ATP, 300 µM UTP and CTP, 20 µM GTP and 5 µCi α[^{32}P]GTP. Transcription extract prepared as described by Manley et al. (14) and Bunick et al. (15) was 30% of the assay volume. Reactions were incubated at 30°C for 60 min (16). Antibodies were added in volumes of either 1 or 2 µl to the transcription extract. After separation the agarose gels were autoradiographed (17).

Production of antibodies to rat nuclear envelope proteins: Antibodies against rat nuclear proteins were produced as previously described (11). They were made against proteins isolated by two-dimensional polyacrylamide gel electrophoresis (against rat lamins A, C and B). The antibodies were raised in chickens and have been characterized for their nuclear localization by immuno-fluorescence and for their specificity by immunoblotting and conform to those described by others (see 18).

Igs were precipitated from sera with ammonium sulfate, dissolved in 0.05 M Tris-HCl, pH 8.2, and dialyzed in three changes of this buffer. This preparation was chromatographed on an ion-exchange column (DEAE-cellulose). The

Ig was dialyzed against borate-saline, pH 8.2, and stored at -20°C. For the incorporation assays, antibodies were inhibitory at a 1:10 dilution. In localization studies (not shown), dilutions of 1:100 gave satisfactory nuclear envelope staining.

Electron microscopy: Permeabilized cells were placed in fixative, 3% glutaraldehyde in 0.1 M Pipes buffer containing 1 mM $MgCl_2$ for 30 min. The samples were postfixed in 1% osmium tetroxide stained overnight in 1% aqueous uranyl acetate at 60°C before embedding in Epon. They were sectioned with a diamond knife and an LKB ultratome, stained with lead citrate and observed on a Zeiss M10 electron microscope.

RESULTS

Electron microscopy of permeabilized cells after antibody treatment: Permeabilized cells prepared from cultures of mouse L-cells after treatment with Ig directed against several antigens were examined under the electron microscope and compared to preimmune Ig-treated control cells. As previously described (13), the detergent treatment of cells left the nuclei intact; however, the internal membrane system was disrupted and thus permeable to antibodies. When permeabilized mouse L-cells are incubated in preimmune Ig (Fig. 1A) and compared to cells that were treated with anti-lamin B Ig (Fig. 1B), the antibodies or the Ig fraction do not appear to disturb the general nuclear structure during the 10-min incubation period.

Transcription of RNA by permeabilized mouse L-cells: Figure 2 shows that the permeabilized cells incorporate uridine monophosphate into TCA-precipitable

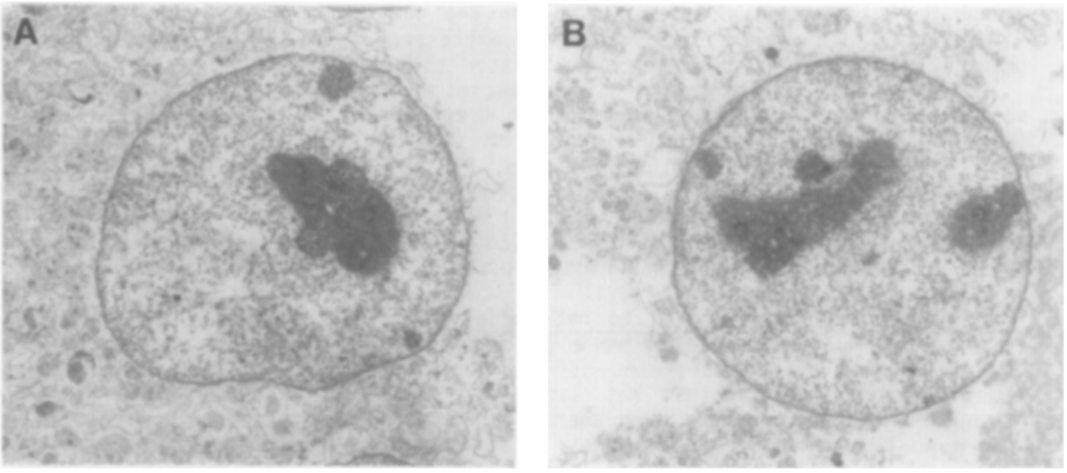

Figure 1: Permeabilized mouse L-cells incubated for 10 min in (A) preimmune chicken Ig, and (B), in chicken Ig including antibodies against rat lamin B. X 8,000

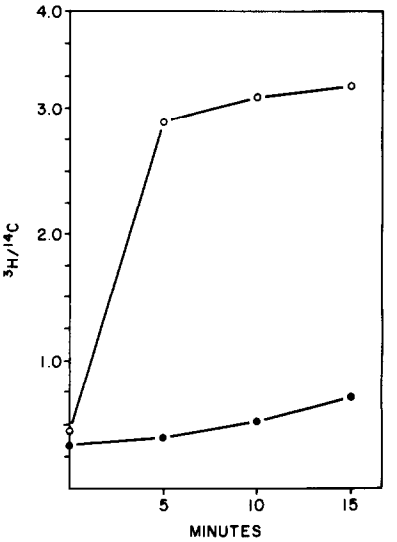

Figure 2: Time course for the Mg^{+2}-dependent RNA polymerase reaction in permeabilized cells incubated at 32°C. Activity was quantitated as cpm [^{3}H]UTP incorporated into RNA per cpm [^{14}C]thymidine incorporated into DNA (o---o). In the presence of NTP, and in the absence of NTP (●---●).

RNA when incubated with the assay mixture described in Materials and Methods. Figure 2 also demonstrates that the reaction is dependent on all four triphosphates. Transcription could be completely inhibited by incubating the permeabilized cells in the presence of α-amanitin. α-Amanitin at a dose of 0.5 µg/ml was sufficient to inhibit RNA synthesis (Table 1).

Transcriptional activity of permeabilized cells after incubation with Ig directed against rat nuclear envelope proteins: Igs directed against lamins A, C, and B were incubated with permeabilized mouse L-cells for 10 min. These antibodies were derived from antigen-containing sections cut out of briefly stained two-dimensional polyacrylamide gels. The antibodies are characterized by immunoblotting and by their ability to block ATP-dependent RNP release as well as NTPase activity (not shown; see ref. 11 for methodology).

Table 1 demonstrates the effect of preincubation of permeabilized mouse L-cells with Ig purified by ion-exchange chromatography on [^{3}H]uridine incorporation into RNA over 10 min. When antibodies directed against lamins B and A, C were incubated with permeabilized mouse L-cells, only anti-lamin B Ig reduced [^{3}H]uridine incorporation to levels of the zero time control,

Table 1. The effect of antibodies directed against lamins A,C and B on the rate of [^{3}H]UTP incorporation in permeabilized mouse L-cells

Conditions	$^3H/^{14}C$
Complete NTP	3.31 ± 0.07
Absence of NTP	0.35 ± 0.05
(0 time) presence of NTP	0.46 ± 0.03
α-Amanitin (0.5 μg/ml)	0.30 ± 0.03
α-Amanitin (1.0 μg/ml)	0.28 ± 0.03
After antibody incubation and in the presence of NTP	
Preimmune Ig	3.32 ± 0.17
Anti-rat lamin B Ig	0.39 ± 0.30
Anti-rat lamin A,C Ig	3.51 ± 0.29

Transcriptional activity was determined as previously described and shown in Fig. 1. Igs were incubated with the permeabilized cells for 10 min at 5°C in 50 mM Tris-HCl and subsequently removed by centrifugation. Incubation mixture was added to the pellets and the activity determined after a 10-min incubation at 32°C. This activity was quantitated as cpm [^{3}H]UTP incorporated into RNA per cpm [^{14}C]thymidine incorporated into DNA. Values are the mean of two determinations performed in triplicate. Ch, chicken-derived antibodies; PIG, preimmune immunoglobulin.

suggesting that the location of lamin B is important in RNA synthesis as measured by our assay.

To test whether antibodies essential for the incorporation of [^{3}H]uridine incorporation could be removed by the antigen, we incubated ~ 0.5 mg/ml of fibrous lamina pore complex proteins with 0.75 mg/ml of the anti-B Ig fraction. The fibrous lamina had been sonicated to disperse this insoluble residue. After high-speed centrifugation to remove the fibrous lamina pore complex and the bound antibody, the remaining Ig was used to test for [^{3}H]uridine incorporation. Table 2 shows that the anti-lamin B Ig fraction absorbed with fibrous lamina pore complex allows nearly as much [^{3}H]uridine incorporation into permeabilized mouse L-cell nuclei as the control. This test also shows indirectly that RNAse does not account for the reduced incorporation to control levels. At the concentration of antibodies used in this

Table 2. Blocking experiment using fibrous lamina pore complex-adsorbed anti-lamin B Ig

Conditions	µg/ml of Ig	$^3H/^{14}C$ 0 min	$^3H/^{14}C$ 5 min	$^3H/^{14}C$ 10 min
+ NTP + preimmune Ig	0.7	0.51	3.41	3.32
- NTP + preimmune Ig	0.7	0.33	0.47	0.66
+ NTP + anti-rat lamin B Ig	0.75 0.25 0	0.48	0.47	0.53 2.82 3.18
+ NTP + anti-rat lamin B Ig adsorbed with fibrous lamin pore complex	0.75			2.94

Aliquots of 10^7 cells/ml were incubated for 10 min with an equal volume of 50 mM Tris-HCl, pH 8.5 and containing immunoglobulin in the concentrations as indicated in the table. After centrifugation the immunoglobulin containing supernatant was removed and the incubation mixture was added. See Materials and Methods.

experiment a four-fold dilution reduced the inhibition to that seen with the fibrous lamina pore complex adsorbed anti-rat lamin B immunoglobulin. Also indicated in Table 2 are the results of 0 and 5 min incorporation of ^3H-UTP.

Whether lamin B was directly involved in RNA synthesis was tested by adding the antibody to the soluble adenovirus RNA transcription assay (15). No inhibition of specific run-off RNA was observed with the anti-lamin B Ig fraction (data not shown). Again this showed that RNAse was not present in our Ig fraction.

DISCUSSION

Using an in vitro transcription assay with whole nuclei, we determined that antibodies directed against a structural nuclear protein strongly inhibit the α-amanitin-sensitive incorporation of [^3H]uridine into RNA. Lamin B is a structural protein of the nuclear matrix (2) or the fibrous lamina pore complex (1). Immunohistochemical methods localize it on the nuclear periphery (1, 18). According to this localization, RNA synthesis as measured in our assay must occur only on DNA directly attached to the fibrous lamina. Fakan et al. (19) have shown by electron microscopy autoradiography that a large part of the new-

ly synthesized RNA can be localized to the perichromatin area of nuclei, that is, at the interphace between the condensed and decondensed chromatin. However, grains were found throughout the nucleus. This argues against a purely nuclear envelope localization of the [^{3}H]uridine incorporation as suggested by inhibition of this incorporation by an antibody to a nuclear envelope-associated antigen, lamin B. Therefore lamin B, or a protein with the same epitope, is either localized throughout the nucleus at concentrations not detectable with the peroxidase- or ferritin-labeled secondary antibodies, or only nuclear envelope-associated RNA synthesis occurs in the in vitro system. Anti-lamin B antibodies not only inhibit RNA synthesis, but as shown earlier, also prevent ATP-dependent RNP release in prelabeled rat nuclei and inhibit NTPase activity (11). It is not clear at present how these inhibitory activities are related. One could speculate that transport away from the site of synthesis is necessary to ensure continued transcription. Any crosslinking of the transport mechanism could thus block RNA synthesis.

The ability of antibodies to prevent or inhibit in vitro functions such as ATP-dependent RNP release, NTPase activity (11) and low-concentration α-amanitin-sensitive RNA synthesis indicates that an insoluble or structural nuclear protein is involved in RNA synthesis. Because RNA synthesis in a soluble system is not affected by anti-lamin B antibodies, it is neither the initiation nor the elongation process that is blocked. Instead, we may have to postulate a regulatory role for the intact nuclear structure which acts at an organizational level different from the actual transcriptional machinery. Models of DNA organization and replication that depend on the nuclear matrix (9, 20) may be drawn upon to explain our finding that antibodies against lamin B can crosslink a structural protein and thereby block RNA synthesis in vitro.

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